Tumor-educated platelets: From RNA to diagnosis

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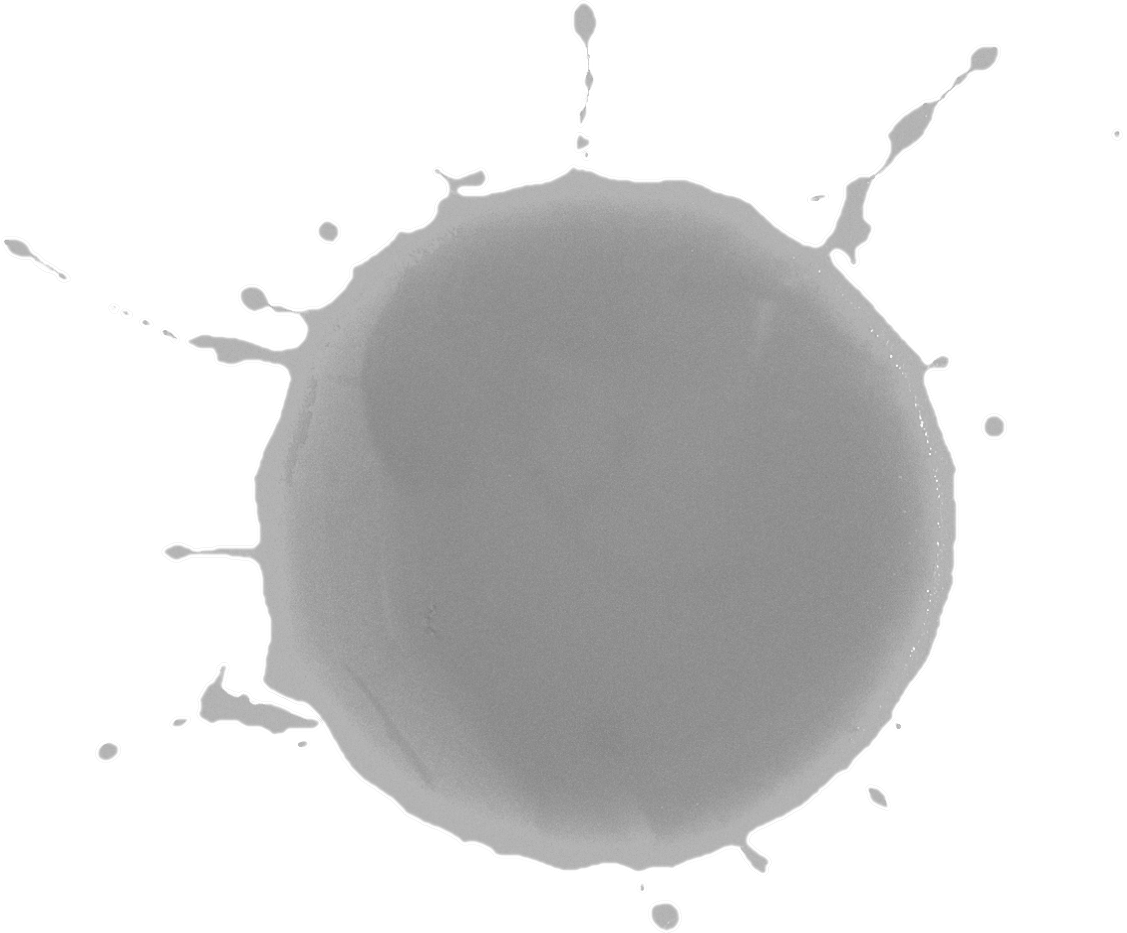
2020



Chapter 1

General introduction and outline of the thesis

Adapted from:

Liquid biopsies in patients with diffuse glioma.

Myron G. Best, Nik Sol, Sebastiaan Zijl, Jaap C. Reijneveld, Pieter Wesseling, Thomas Würdinger. Acta Neuropathologica 2015 Jun;129(6):849-65

Platelet RNA as a circulating biomarker trove for cancer diagnostics.

Myron G. Best, Adrienne Vancura, Thomas Würdinger. Journal of Thrombosis and Haemostasis 2017 Jul;15(7):1295-1306

Tumor-educated platelets as a noninvasive biomarker source for cancer detection and progression monitoring.

Myron G. Best, Pieter Wesseling, Thomas Würdinger. Cancer Research 2018 Jul 1;78(13):3407-3412

'A stage-shift in one drop of blood: early detection of cancer'.

Myron G. Best. TEDx Groningen 2016

'Cancer is one of the most devastating and invalidating diseases known. The disease is caused by aberrant growth and proliferation of malignant cells with the devastating potential to spread throughout the body. Currently, each year worldwide, 18 million people are diagnosed with cancer[1], of which a majority has at moment of diagnosis a stage IV, highly spread and untreatable tumor. These numbers are, unfortunately, still increasing. Screening of blood on a regular basis can be the difference between life and death, treatable stage I and untreatable stage IV cancer.

In 1971, president Nixon of the United States declared the symbolic War On Cancer. By raising research funds, he initially aimed at a cure for this devastating disease. Till today, we didn't make it to cure all types of cancers, even not with our current arsenal of treatment options, such as surgical removal, chemotherapy, radiotherapy, and the recently introduced immunotherapy, Still, it doesn't do what we want it to do. It seems that this initial plan - plan A: 'Hit the tumor as hard as you can' - fails. Obviously, tumors hide like phantoms. Small entities, peanut-sized clumps of cells, in development towards a large and widely spread tumor. Thus, early detection of such a clumb, while the tumor and the phantom is not extensively spread yet, would simplify treatment significantly.'

Currently, cancer is diagnosed by clinical presentation, radiology, biochemical tests, while for definitive diagnostics pathological analysis of tumor tissue is generally required. Due to the increasing number of cancer screening tests, for example by mammography and low-dose computed tomography lung imaging, tumors can be detected at an earlier stage while patients are still asymptomatic. Following, clinical oncology practice relies on (repeated) removal of tumor tissue through biopsies for analysis of tumor-linked genetic alterations and other cancer biomarkers. Although tumor tissue biopsy is the current gold standard for cancer diagnosis and represents an essential tool in cancer management, it has become clear in recent years that the information acquired from a single biopsy provides a spatially and temporally limited snapshot of a tumor and often fails to reflect the heterogeneity of the disease. Moreover, the fact that tumor biopsies are invasive poses a limitation for repeated sampling[2].

It has been known for decades that blood is a rich source of tumor-associated biomarkers, which can be isolated from several biosources[2,3]. These include the mono-nuclear cell fraction, encompassing leucocytes, circulating tumor cells (CTCs) and circulating endothelial cells (CECs), and plasma and serum, which contain extracellular vesicles (EVs), cell-free DNA (cfDNA) and RNA (cfRNA), plasma proteins and metabolites, and tumor-educated platelets (TEPs)[3]. These biomolecules and

biosources are being regarded as part of the pro-tumorigenic and systemic activity of the primary tumor (e.g. CTCs, CECs, EVs, and TEPs) or are considered to derive from merely passive release during tumor cell apoptosis and necrosis (e.g. cfDNA and cfRNA). It has been shown that each biosource and biomolecule in blood has its potential for blood-based cancer diagnostics, companion diagnostics, prognostics, and therapy monitoring. For example, analysis of cfDNA can be employed for detection of point mutations or structural variants but also of copy-number aberrations, differential cfDNA length, and methylation status. Furthermore, EVs have been shown to harbor proteins and RNA biomarker molecules of variable length, but also surface membrane proteins that are correlated to organ tropism for cancer metastasis[4]. TEPs are considered as local and systemic responders to the presence of cancer[5], thereby sequestering EV-derived RNAs[6–8] and proteins[9], as well as altering their spliced RNA profile[10–13]. Interestingly, a combined readout of multiple biosources and biomolecules, here plasma-derived cfDNA and proteins, might leverage the information included in each individual source[14], resulting in the detection of 62% of stage I-III cancers with >99% test specificity, and providing an innovative approach for detection of cancer in an early stage. In all, liquid biopsies may enable 1) early detection of cancer (screening), 2) prognostication for the individual patient by providing information about stage and spread of the disease, 3) identification of new targets for personalized treatment, 4) pre-treatment therapy response prediction, 5) early therapy response monitoring and 'real-time' assessment of treatment effectiveness, and 6) early detection of recurrence of the disease.

Blood platelets

Platelets originate from megakaryocytes in the bone marrow and are second to red blood cells as the most-abundant cell type in peripheral blood. Healthy individuals have 200-500 million platelets per milliliter of whole blood, although the size of the platelet population can increase or decrease during infection, cancer, or bone marrow disease[15,16]. Cardiovascular diseases (CVD) are currently the leading cause of morbidity and death (23.5%) in first world countries such as the United States, closely followed by cancer (22.5%)[17]. Both diseases are caused by aberrant pathophysiological processes in cells and tissues, including local and systemic inflammation, and share the contributive pathological value from circulating blood platelets[18,19]. In cardiovascular disease, several vessels such as the coronaries of the heart or major arteries in the brain are prone to formation of blood clots[20]. Upon subendothelial presentation of collagen, platelets activate and support thrombogenesis cascades. Additional platelets are activated, sequestered, and

cross-linked with other activated platelets via e.g. glycoprotein IIb/IIIa in the fibrin clot[21]. Thereby, platelets initiate fatal events causing cardiovascular-related deaths[20,21]. Platelets also 'scan' the blood stream for pathogens[22], and subsequently activate circulating immune cells[23]. In addition, platelets are involved in inflammatory conditions such as rheumatoid arthritis[24,25], diabetes[26], pulmonary hypertension[27], HIV[28], and Alzheimer's Disease[29,30]. Previously, platelet transcription factors, such as PPAR**γ**, RuvB-like 2, STAT3, and STAT5A, were shown to regulate inflammatory processes[31]. Also in non-human species, thrombocytes have been also linked to the immune system. Novel insights from nucleated chicken thrombocytes highlight their involvement in T cell activation through MHC-II molecules, thereby modulating the adaptive immune system[32]. Although, anucleated human platelets lack MHC-II protein expression, they were shown to promote T cell responses through CD40L molecules *in vitro*[33]. These findings suggest that human platelets are involved in innate and adaptive immunity.

In 1968 it was reported that the enzyme neuroaminidase reduced the number of TA3 breast cancer cell-induced tumor metastases caused by intravenously co-injected tumor cells[34]. This was associated with thrombocytopenia and provided support for a model in which platelet aggregation and tumor embolization enhance tumor metastasis[35]. More recent experiments have shown that platelets can promote epithelial-mesenchymal transformation (EMT; a hallmark of cancer enabling cells to extravasate their primary environment and spread via the blood stream). Platelets accomplish this by release of TGF-**β** and direct physical platelet-tumor cell interaction thus activating intracellular NFkB-signaling[36]. Furthermore, platelets can physically cover circulating tumor cells, forming clots, and present MHC-I molecules, thereby mimicking these platelet-tumor clots as 'self'[37,38]. In addition, platelets can prepare metastatic niches by releasing pro-angiogenic factors[9,39], recruitment of granulocytes[40], and remodeling of bone for tumor cell seeding[41]. Again, platelets provide support to the metastatic disease process[5], thereby significantly reducing patient survival, and often rendering these patients incurable.

Nucleic acids in blood platelets

Circulating platelets were shown to interact with various cells and molecules in the blood stream and their transcriptome is altered in response to extracellular queues (**Figure 1**). Interestingly, platelets contain multiple RNA species[42–46], and the presence of nucleic acids in platelets and the ability of platelets to translate mRNA into proteins has been known for a long time[47–49]. After introduction of high-throughput characterization methods, such as micro-arrays and RNA-sequencing techniques, a much deeper insight on the entire RNA repertoire has been explored,

including the discovery of small and longer regulatory RNAs[42,44,50–54]. So far, several RNA families have been identified in platelets, including precursor and spliced mature mRNAs (pre-mRNA and mRNA), ribosomal RNA (rRNA), microRNAs (miRNAs), small nuclear (snRNA), small nucleolar RNA (snoRNA), antisense RNAs (asRNA) and transfer RNA (tRNA)[42–46,51,55] (**Table 1**). It is important to note that the presence of a particular RNA family in platelets does not imply that this molecule is functionally active and/or the function is similar as in nucleated cells. Advances in molecular analysis techniques allow for the high-throughput profiling of platelet RNAs from relatively small blood volumes, e.g. 3 mL citrate blood[56,57], or even from the equivalent of a single drop of blood (which equals approximately 50 µL of whole blood)[10]. Quantification of the RNA content in platelets indicated that a single platelet can contain an estimated ~2.2 fentogram of RNA[58]. It has been noted that younger, reticulated platelets have a 20-40-fold enriched total RNA concentration, as measured by RNA-binding thiazole orange staining[59]. This suggests that younger platelets contain more (unspliced) RNAs that might be subjected to decay or released while platelets circulate or become activated[59,60]. The presence of a functional spliceosome - required for splicing of pre-mature mRNA into mature mRNA molecules for protein translation, and previously thought to be present in only nucleated cells – was somewhat unexpected[45]. The enriched levels of RNA in younger platelets and the presence of a splicing machinery suggests that this mechanism might serve as a gatekeeper for pre-mRNA processing and protein translation.

Platelets lack a nucleus, hence, no genomic DNA is available for transcription of new RNA molecules. However, platelets do contain mitochondrial DNA, which might be subjected to transcription upon activation[61]. RNA in newly generated platelets is inherited from the megakaryocyte. The RNA transcription in megakaryocytes is regulated by transcription factors[62], and RNA molecules are actively allocated into the pro-platelets during thrombopoiesis[63]. By careful comparative analysis between megakaryocyte and platelet RNA molecules coding for matrix metalloprotease (MMP) and tissue inhibitor of metalloprotease (TIMP), Cecchetti et al. proposed that specific RNA transcripts are sorted into platelets[63] (**Figure 1**). The authors hypothesized that this sorting mechanism is similar to a sorting mechanism observed in neuronal dendrites[64]. Several proteins, such as CASC3, STAU1, STAU2, EIF4A3, implicated in mRNA transport in neurons were shown to be expressed in platelets[63], and platelets are considered to be rich reservoirs of the neurotransmitter serotonin[65]. This suggests that megakaryocytes might exhibit similar sorting mechanisms for mRNA allocation as observed in neurons.

The most studied type of RNA in platelets is mRNA. mRNAs exhibit a conserved setup containing a cap structure at the 5'-end of the sequence, followed by a 5'-untranslated region (UTR), a stretch of exonic and intronic sequences, a 3'-UTR, and a poly-A tail (**Figure 2**). The cap at the 5'-end and the poly-A tail serve as markers for mRNA stability, whereas both UTRs contain sequences for RNA-binding proteins and miRNA-mediated expression regulation. During the maturation process, intronic stretches in pre-mRNAs are subjected to splicing, resulting in mature mRNA transcripts.

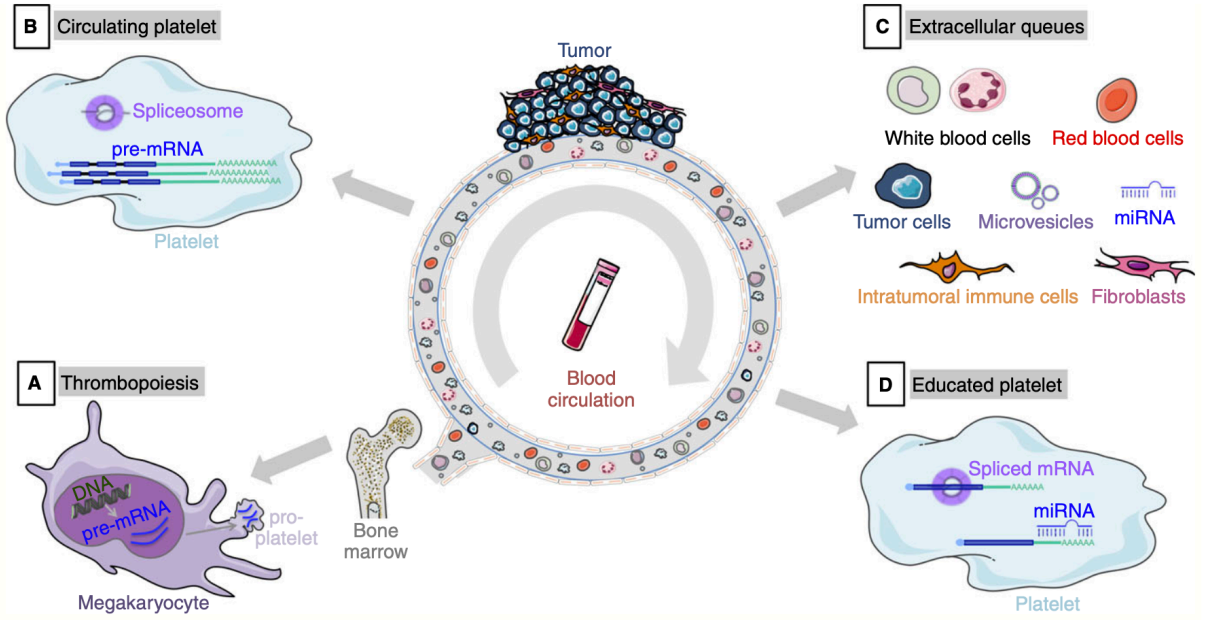


Figure 1 – Schematic representation of tumor-mediated education of the megakaryocyte and platelet compartment.
Megakaryocytes, platelet pre-cursor cells, are localized in the bone marrow and sort specific RNA and proteins into pro-platelets during thrombopoiesis (A). Race as well as gender influence the megakaryocyte transcriptome and can thereby influence the platelet RNA content. Platelets are released from the bone marrow into the circulation. Circulating platelets contain a variety of RNA transcripts and proteins, such as mRNAs and spliceosomal proteins (B). Presence of a tumor at a certain place in the circulation (top) near a vessel initiates platelet education. In addition, neighboring and distant cells continuously signal via proteins and vesicles to the platelets (C). Here, platelets can be supplemented with proteins and RNAs transported by lipid vesicles, and processed intracellularly. Of note, platelets also release molecules while circulating in e.g. platelet microparticles. Following queue-mediated intra-platelet activation, regulatory RNAs and proteins induce a platelet specific response to the queues resulting in specific platelet mRNA splicing programmes (D). These RNAs can potentially be used for platelet-based disease biomarker detection.

RNA family	Abbreviation(s)	Definition or function	References
messenger RNA	mRNA	RNA coding for proteins	Agam *et al.* 1976, Booyse & Rafelson, 1967, Rowley *et al.*, 2011, Bugert *et al.*, 2003, Bray *et al.*, 2013, Gnatenko *et al.*, 2003, McRedmond *et al.*, 2004, Kissopoulou *et al.*, 2013
circular RNA	circRNAs	Possibly miRNA sponge	Alhasan *et al.*, 2016
ribosomal RNA	rRNA	Possibly regulation of translation, protein synthesis	Bray *et al.*, 2013
small nuclear RNA	snRNA	Possibly RNA splicing	Bray *et al.*, 2013, Denis *et al.*, 2005
small nucleolar RNA	snoRNA	Possibly RNA modifications	Bray *et al.*, 2013, Denis *et al.*, 2005
transfer RNA	tRNA	Possibly protein translation	Bray *et al.*, 2013
micro RNA	miRNAs	Post-transcriptional regulation	Landry *et al.*, 2009
antisense RNA	asRNA	Possibly destabilization of mRNA and of inhibition translation	Bray *et al.*, 2013

Table 1 – RNA families identified in platelets.
Table 1 summarized the RNA species that have been identified in platelets and their general definition or function (in nucleated cells). Official abbreviations of the RNA families are noted in a separate column.

According to multiple studies exploiting the technical potential of several high-throughput characterization methods, transcripts and multiple alternative splicing isoforms of roughly a third of all human genes (~5000-9000 genes) were detected within platelets[10,42,50,51,53,54,61]. To uncover the potential functional processes deduced from mRNA expression data, several gene ontology (GO) software modules are available, such as DAVID, Panther and GOrilla. The GO analyses, using mRNA expressed in platelets of healthy individuals, indicated that detected genes are functionally enriched for biological processes such as coagulation, platelet degranulation, cytoskeletal dynamics, receptor binding, secretion and G-protein signaling[10,51,54,66] (**Figure 1**). These biological processes are closely related to well-known phenotypic activities of platelets, such as the involvement of cytoskeletal proteins during platelet activation[67], although functional studies are frequently lacking. In addition, robust detection of genes depends on the technical approach (RNA-seq versus micro-array versus RT-PCR) and the depth of analysis (coverage). As these platelet RNA profiling studies have been performed on a natural mixture of platelets ('bulk platelet pellet analysis'), including both younger and older platelets, it is unlikely that the platelet RNA pattern is consistent among all individual platelets, though evidence for this is lacking.

Platelets exploit a functional spliceosome for pre-mRNA splicing
Platelets possess the ability to translate mRNA into proteins, as was demonstrated for B-cell lymphoma 3 (BCL3) and the inflammatory cytokine interleukin-1**β** (IL-1**β**)[68,69]. It was shown that resting platelets contain primarily non-spliced premature IL-1**β** mRNA transcripts[45]. This may indicate a broad post-transcriptional

regulatory mechanism of platelet gene expression, which employs a functional splicing machinery to process pre-mRNA transcripts into an intronless translatable mRNA transcript[69]. Indeed, it has been shown that quiescent platelets contain many proteins associated with the spliceosome, including U1 70K, U2AF, SRm160, SMN, and SF2/ASF (**Figure 2**). Small nuclear RNAs (snRNAs) U1, U2, U4, U5, U6, that bind to pre-mRNA, are present as well[45]. Upon activation of quiescent platelets using thrombin on a fibrinogen adherent layer, the activated platelets splice IL-1**β** pre-mRNAs into mature mRNA transcripts, resulting in the synthesis of IL-1**β** proteins[45]. An additional study showed the splicing initiation of IL-1**β** following Akt and Jun phosphorylation[70].

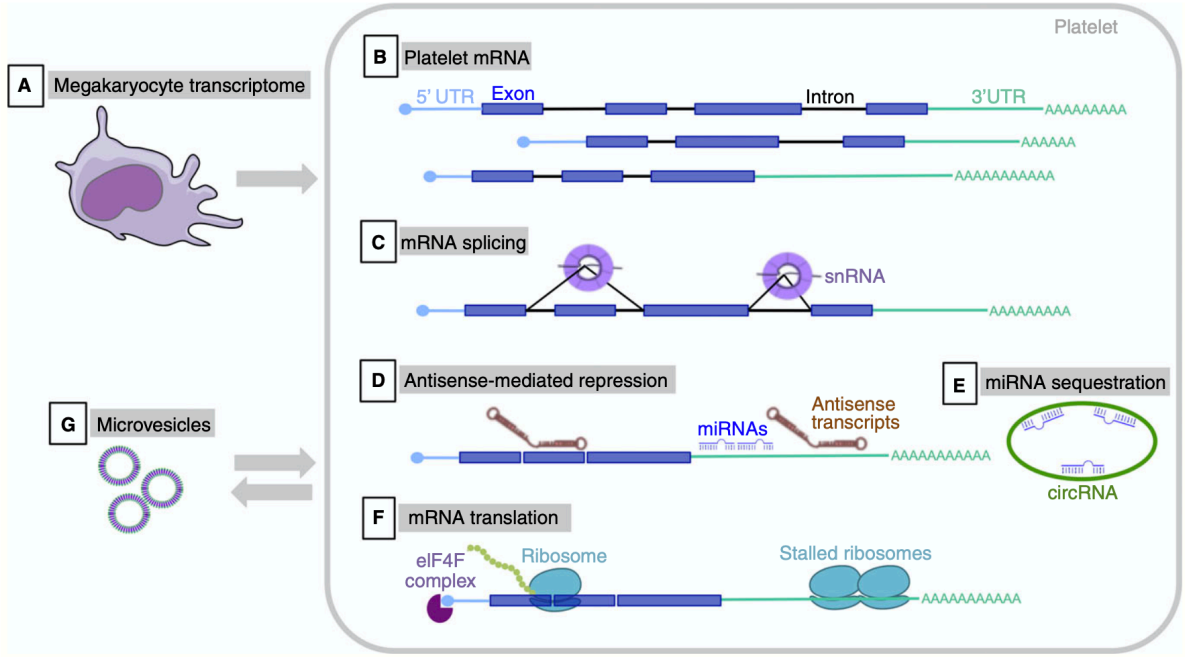


Figure 2 – Comprehensive overview of regulatory RNA molecules in platelets.
The megakaryocyte sorts distinctive RNA molecules into platelets during thrombopoiesis (A). The mRNA transcripts found in platelets contain 5' cap structures, 5'-UTRs, exons, introns and 3'-UTR sequences (B). These mRNA transcripts can be spliced with an intact spliceosome machinery, containing snRNA molecules and spliceosomal proteins (C). Regulatory RNA such as antisense RNA and miRNAs can repress mRNA molecules (D). These antisense RNAs can be sequestered by circRNA (E). Mature mRNA transcripts can be translated, whereas the eIF4F complex binds to the 5'-end to induce ribosome attachment. Ribosome stalling, due to inhibited ribosome recycling processes, influences the mRNA translation process (F). Platelets can release or sequester microvesicles containing various RNA molecules (G).

Splice-regulation was also demonstrated for platelet tissue factor (TF), which initiates the coagulation cascade. The TF pre-mRNA was spliced upon stimulation of platelets with bacteria, staphylococcal alpha-toxin, or lipopolysaccharide. Initiation of TF splicing was shown to be mediated by cdc2-like kinase (Clk1) phosphorylation, which could be inhibited by co-incubation with the Clk1-blocking benzothiazol compound Tg003, and subsequent phosphorylation of the RNA-binding protein SF2/ASF (SRSF1)[71].

Although the spliceosome in nucleated cells usually acts co-transcriptionally, the cytoplasmic origin of a spliceosome in platelets remains unclear. The translation machinery, consisting of rRNA and ribosomal proteins (e.g. S6 and ribosomal P antigen proteins[72]), amino acid-transporting tRNAs, and rRNA-modifying snoRNAs, was shown to be active in platelets[45,49,73]. snoRNAs accumulate in the nucleolus of nucleated cells and are involved in translation and splicing as they chemically modify nucleotides of rRNA and snRNA molecules (e.g. methylation and pseudo-uridylation[74]). Interestingly, snoRNAs have also been detected within anucleated platelets[51,75]. Novel snoRNA functions were recently described in nucleated cells[76], and further comparative studies with platelets could be of interest. Processed fragments of the snoRNA SNORD 115 were shown to influence the alternative splicing of the serotonin receptor 2C pre-mRNA by its sequence complementarity and binding to splice factors in order to influence splicing[76]. The presence of both serotonin receptor 2C pre-mRNA and splice factors (e.g. U1 70K and U2AF[45]) in platelets might indicate that the snoRNAs in platelets mediate alternative splicing (**Figure 2**).

Regulatory RNAs interact with platelet mRNA transcripts

Platelets contain pre-mRNAs that can be processed into mature mRNAs by splicing after an initiating event such as cytokine-mediated receptor activation or initiation of the hemostatic pathway[68,69]. A second mRNA-maintaining regulatory mechanism is provided by regulatory RNAs, such as the ~22 to 24 nucleotide (nt) long miRNAs. miRNAs are bound to the RISC complex, which can mediate gene silencing through the RNA interference (RNAi) pathway[77] (**Figure 2**). The nuclear miRNA processing proteins Drosha and DCGR8 were shown to be absent in platelets but present in megakaryocytes[44]. However, it was reported that platelets exhibit a functional miRNA processing pathway, including the pre-miRNA-processing proteins Dicer and Ago2, and a diverse repertoire of miRNA molecules[44,55,78,79]. Specific miRNome changes are observed during platelet activation using thrombin and suggest mediation of platelet response to activating stimuli[78]. Ago2 protein levels were shown to be increased during platelet activation, indicating a higher miRNA

regulatory activity, and thus possibly affecting the platelet proteome[78]. More than 500 different miRNAs have been detected in human platelets[44,55], and are suggested to be involved in myeloid cell differentiation, megakaryocytopoiesis and thrombopoiesis[55]. It has been estimated that the 15 most abundant miRNAs encompass 90% of the miRNAs repertoire[80]. It has also been suggested that miRNAs are altered in blood of patients with diabetes mellitus type 2, and cultured megakaryocytes respond to hyperglycemic conditions by changing miRNA patterns[81] (**Figure 1**). Diversity in the platelet miRNA landscape is shaped by 3'-terminal end modifications. Such modifications, including 3'-end adenylation or uridylation by terminal nucleotidyltransferases, affect miRNA biogenesis and stability, with potential functional implications[55,82,83]. These terminal modifications can potentially influence mRNA target silencing efficiency, for example by decreasing the miRNA incorporation into Ago2 protein complexes[55]. During miRNA-mediated repression, the miRNA molecules act through recognition of specific binding sites, usually located in the 3'-UTR of an mRNA[84]. Bray et al.[51] described the presence of longer (>24 nt) antisense RNA transcripts, which are coding in antisense to the 5'- or 3'-UTR or a full-length exonic sequence of protein-coding transcripts. These antisense RNAs destabilize mRNAs and repress translation[85], and potentially compete for mRNA binding sites by masking the miRNA binding sites[43,51]. The biological functionality of these antisense RNAs in platelets has not yet been demonstrated. Analyzing gene-flanking reads indicated that genes coding for mRNAs in platelets harbor 3'-UTR sequences twice as long as those of nucleated cells (1,047 bases in platelets versus average 492 bases in nucleated cells)[43,84]. These 3'-UTRs are involved in stabilizing mRNA molecules and contain potential binding sites for regulatory miRNAs[84] and RNA-binding proteins[86]. miRNA target prediction tools predict that the platelet-specific 'elongated' 3'-UTRs harbor additional miRNA binding sites[43]. This suggests higher miRNA regulatory activity on the elongated 3'-UTR mRNA transcripts in platelets as compared to the shorter 3'-UTRs in nucleated cells. During platelet activation, it was shown that at least six miRNAs were either up- or down-regulated in response to thrombin, a platelet activation molecule[87]. These findings suggest that the miRNA repertoire alters due to signal inputs and induces an agonist-specific platelet response[87,88].

Circular RNA (circRNA) has been identified as a potential regulatory RNA molecule in platelets. circRNAs are generated from mature mRNAs by exonic back-splicing mediated by the spliceosome[46,89]. During back-splicing two exons that are usually joined by linear splicing are connected in a back-ward direction, resulting in a circular RNA molecule. A specific sample preparation protocol, with enzymatic RNAse R-mediated degradation of linear RNAs, while maintaining circRNAs,

enabled the identification of back-spliced exon junctions from RNA-sequencing data, and allowed for profiling of circRNA[46]. Alhasan et al. reported that human platelets and erythrocytes are highly enriched for circRNAs (>17-188 fold-enrichment as compared to several types of nucleated cells)[46]. Up to 3,162 genes in platelets were identified to be enriched in circRNAs. The circRNAs are hypothesized to be generated in platelets as an RNA back up storage pool, protected from decay[59] (**Figure 2**). Synthesis of circRNAs in the platelets themselves is more likely than their inheritance from megakaryocytes during platelet birth, since higher levels of circRNA were detected in platelets as compared to cultured megakaryocytes[46]. However, the stability of circRNAs in cultured megakaryocytes remains unknown. In addition, circRNAs may also behave as miRNA sponges, thereby sequestering regulatory miRNAs[90,91]. The circRNAs can show sequence complementarity to certain miRNA sequences, which may potentially compete with mRNA-miRNA binding sites[90]. In neuronal tissue the human circRNA CDR1as, which has a complimentary sequence to the cerebellar degeneration-related protein 1 (CDR1) transcript, was shown to densely bind and inhibit miR-7 molecules[91]. This circRNA-mediated mechanism might act in a similar fashion to the miRNA-mediated RNA silencing process in platelets[92]. The platelet mRNA content appears to be regulated at multiple levels and is a highly dynamic process. Analyzing RNA fragments and ultimately better understanding the regulation of platelet gene expression in healthy and disease could lead to novel biological insights.

Platelets allow for mRNA translation

Platelets have the ability to translate mature, spliced RNAs into proteins[53,93,94]. Several studies reported extensive protein profiles of platelets, however, it seems that not all platelet mRNAs are translated. Londin et al. reported that for almost 60% of detected mRNAs no corresponding protein was identified[94,95]. In addition, Alhasan et al. suggested that the rapid degradation of spliced or non-spliced linear RNAs provides a potential explanation for the low correlating platelet RNA and protein profiles[46,60] (**Figure 2**). RNA molecules, for which no protein was detected in platelets, may either be megakaryocyte-specific or the protein products may have been released to vascular locations via exosomes or microparticles or the transcripts are translationally silenced until receiving specific inputs, e.g. inflammatory responses or clotting induced subsequent protein expression[94]. Of note, for analysis of platelet nucleic acids and protein content, high-purity platelet preparations are required, since contaminating nucleated cells or remaining plasma proteins can influence the resulting profiles significantly. The exact regulatory mechanism regulating platelet protein translation is unknown. It has been shown that the

regulation of ribosomes can influence the protein translation rate within platelets. Mechanistically, platelet RNA seems to be primarily bound to polysomes[69]. To prevent instant initiation of protein synthesis, the 5' mRNA cap-binding protein eukaryotic initiation factor (eIF) 4E (eIF4E, a central component of the eIF4F translation initiation complex), present in platelets[96], is physically separated from unprocessed RNAs[97]. The platelet eIF4E protein is localized in the membrane skeleton and soluble fraction, whereas platelet mRNA transcripts are bound to the cytoskeletal core[97]. This physical separation is overcome after platelet activation when eIF4E proteins are redistributed to the cytoskeleton for increased interaction with mRNA molecules and result in downstream protein synthesis. In addition, ribosomes need to be recycled by several distinct proteins and intracellular mechanisms to be reused for further mRNA translation. A recent study revealed a surprising accumulation of ribosomes on the 3'-UTRs of mRNA transcripts in platelets[98]. These accumulated ribosomes were shown to result in the natural loss of specific ribosome-recycling factors during thrombopoiesis. The availability of ribosome-recycling factors was hypothesized to be critical for maintaining the pool of available ribosomes, and thus crucial for translational activity in platelets[98]. In conclusion, control of initiation of platelet RNA splicing and protein translation is thought to be regulated by spatial and temporal interactions between platelet RNA transcripts and multiple platelet RNA-binding proteins, such as eIF4E[97] and SRSF1[71] (**Figure 2**).

Platelet RNA profiles are dynamic and transient

Platelets are loaded with premature mRNA transcripts before budding from the megakaryocyte. During platelet circulation the platelet RNA seems to be subjected to decay[60], as observed by a reduced thiazole orange signal[59], and possibly stored in circRNA products[46]. Upon platelet activation, pre-mRNA can be spliced and subjected to protein synthesis. Platelets locally and distantly communicate with the environment via 1) direct physical interactions (e.g. binding to glycoprotein IIb/IIIa and p-selectin), 2) secretion and horizontal transfer of platelet-derived microparticles[99–103], and 3) release of chemokines and cytokines[23,104] (**Figure 1**). Vice versa, platelets are able to capture and sequester circulating proteins and vesicles from their surroundings[6,9,39]. Platelet-derived extracellular vesicles (EV) or microparticles are the most abundant vesicles in the blood stream[102]. It has been shown that the cargo and total number of platelet-derived EVs can change by exposing platelets to LPS or calcium *in vitro*, thus activating the platelets[105]. Particles from thrombin activated platelets allowed for the delivery of mRNA regulatory Ago2-miRNA complexes to endothelial cells[103]. Platelet microparticles were shown

to deliver functional miR-126-3p molecules to macrophages, altering their gene expression and reprogramming those immune cells[102]. In addition, platelets sequester molecules, including RNA. It has been reported that tumor-derived EVs transfer tumor-specific RNAs *in vitro* and *in vivo* to circulating platelets[6,7,10]. Sequestration and release of RNA molecules may alter the platelet transcriptome and induce downstream processes such as RNAi or alternative splicing (**Figure 2**).

It appeared that platelet RNA profiles can be associated with demographic variables, such as age, gender, and race[10,66,106]. In black individuals, an altered platelet reactivity upon PAR4 thrombin receptor-mediated activation and initiation of platelet aggregation was observed[106]. Specific single nucleotide polymorphisms in the PAR4-gene and differential levels of miR-376c in megakaryocytes of black individuals were associated with elevated levels of PCTP RNA[106,107] and enhanced intra-platelet Gq-signaling[107]. It also appeared that the age of the individual is associated with the platelet RNA profiles. At least 129 non-mitochondrial mRNAs and 15 miRNAs were differentially expressed in platelets of younger individuals as compared to older individuals (range: 18-46 years old), and the expression of mitochondrial RNAs was inversely associated with age[66]. Notably, at the level of the bone marrow, platelet-biased hematopoietic stem cells (HSC) modulate platelet production under control of thrombopoietin levels[108]. Aging of individuals is associated with a 50-fold increase of platelet-biased hematopoietic stem cells, possibly at the cost of lymphoid cell production[109]. These studies suggest that experimental results regarding platelet biology and biomarker discovery and validation should be interpreted in well-specified and matched cohorts. The dynamics of platelet RNAs are summarized in **Table 2**.

Process	Mechanisms	References
Thrombopoiesis	Megakaryocyte transcriptome and (specific) RNA sorting and packaging into platelets.	Plé *et al.*, 2012, Cecchetti *et al.*, 2011
Stem cell aging	Megakaryocyte transcriptome and bone marrow-derived queues	Grover *et al.*, 2016
Platelet activation	Thrombin	Denis *et al.*, 2005, Cimmino *et al.*, 2015
RNA splicing	Alternative splicing, possibly mediated by RBPs	Denis *et al.*, 2005, Rondina *et al.*, 2011, Schwertz *et al.*, 2006
RNA decay	Alternative splicing, time-dependent	Alhasan *et al.*, 2016, Schubert *et al.*, 2014, Angénieux *et al.*, 2016
RNA interference	miRNA- or asRNA-induced RNA degradation	Landry *et al.*, 2009, Plé *et al.*, 2012, Cimmino *et al.*, 2015,
Translational activity	Presence of ribosomes, availability of ribosome recycling factors, RBPs	Angénieux *et al.*, 2016; Mills *et al.*, 2016
RNA uptake	Vesicle sequestration	Nilsson *et al.*, 2011, Nilsson *et al.*, 2016, Best *et al.*, 2015
RNA release	Vesicle release and intracellular transfer	Laffont *et al.*, 2013, Kirschbaum *et al.*, 2015
RNA modifications	TUT4/GLD2 (nucleotidyltransferases), snoRNAs	Plé *et al.*, 2012, Bray *et al.*, 2013, Denis *et al.*, 2005

Table 2 – Dynamics of platelet RNA.

Table 2 summarizes biological processes that might dynamically affect the platelet RNA content. Indicated are the biological process, the mechanism, and the study in which the process has been studied. RBPs; RNA-binding proteins.

Recently, blood platelets have entered the array of blood-based biosources[110,111] (**Figure 1**). For blood-based cancer diagnostics and prognostics in multiple tumor types and stages, reports dating back decades describe the potential diagnostic use of platelet counts[112–118], platelet size[114,119], and platelet protein markers such as p-selectin[115,120–124], platelet factor 4 (PF4)[125,126], thrombospondin[127] and thrombopoietin[128,129]. Several studies have measured alterations in platelet counts and the ratio of reticulated young 'RNA-rich' platelets to the total platelet population, showing changes after tumor treatment following surgery[122,130,131], and chemotherapy[118,132]. Functionally, tumor growth has been related to platelet hyper-reactivity[133], although this was not further confirmed in a recent study investigating platelets collected from patients with ovarian cancer[134]. Also, previous studies have identified diagnostic platelet RNA signatures for cardiovascular abnormalities[135–139], inflammatory conditions[18,140], sickle cell disease[141,142], essential thrombocytosis[143,144], and cancer[6,10,12]. In 2010, Calverley et al. used micro-array analysis to profile the platelet mRNA of seven healthy individuals and five metastatic lung cancer patients[12]. The platelet samples of the lung cancer patients were collected prior to any treatment, and 200 altered RNAs were discovered between the healthy individuals and the lung cancer patients, of which 197 genes (99%) were decreased in platelets of lung cancer patients. This study also identified the presence of alternative mRNA splicing mechanisms in platelets of lung cancer patients as compared to healthy individuals, and proposed that platelet RNA might have a predictive role for the detection of metastatic lung cancer[12]. It should be noted that these explorative studies have been performed on small cohort sizes, largely excluding individuals with inflammatory or other benign conditions, and require thorough validation using larger datasets.

In this thesis I investigated the potential of TEPs for RNA-based liquid biopsies. **Chapter 2** presents a proof-of-concept study establishing the potential of TEP-derived RNA-profiles for blood-based pan-cancer, multiclass, and molecular pathway cancer diagnostics, using a novel pipeline termed 'thromboSeq'. Though this study was critically evaluated by us and other, a follow-up study described in **Chapter 3** addressed the concerns and presented an improved version of the thromboSeq-protocol, focusing on the detection of non-small-cell lung cancer. In order to enable researchers world-wide to employ, test, and improve the thromboSeq-pipeline, the full wet- and dry-lab protocol has been published in detail, as described in **Chapter 4**. To investigate the value of thromboSeq for the earliest detection of cancer a follow-up study described in **Chapter 5** has been conducted in which detection of breast cancer in its earliest stage is tested. Ultimately, the thromboSeq-pipeline has

been tested for the diagnostic accuracy of brain tumors, which are notorious for their low traces in blood, as described in **Chapter 6**. Here, also the dynamics of tumor-educated platelets RNA-profiles during adjuvant chemoradiotherapy is being investigated. **Chapter 7** closes this thesis with a thorough discussion regarding the field followed by an overview of future perspectives.